SULFUR MUSTARD- AND PHOSGENE- INCREASED IL-8 IN HUMAN SMALL AIRWAY CELL CULTURES: IMPLICATIONS FOR MEDICAL COUNTERMEASURES AGAINST INHALATION TOXICITY

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ABSTRACT

Inflammation and edema are associated with respiratory and cutaneous exposure to sulfur mustard (SM) as well as with phosgene-induced lung injury. IL-8 is a key inflammatory cytokine that recruits neutrophils linked with the onset and progression of acute lung injury caused by inhalation of these chemical agents. In the present study, human lung small airway cell (SAC) cultures were exposed to either SM [25 to 400 μM] or phosgene [0.1 to 6.4 ppm • min]. IL-8 was increased after exposure to either SM or phosgene. At the optimum exposures for SM (100 μM) and phosgene (1.6 ppm • min), IL-8 was increased by 1013 ±123 pg/ml and 965 ±181 pg/ml, respectively. Higher exposures to either agent increased cytotoxicity and decreased IL-8 levels. Ibuprofen has shown efficacy against phosgene pulmonary toxicity in mice. Ibuprofen (62, 125, 250, 500, 1000 μM) significantly diminished phosgene-increased IL-8 in SAC cultures exposed to 2 ppm • min phosgene. Maximum inhibition of nearly 50% of phosgene-increased IL-8 was seen at 125 and 250 μM doses of ibuprofen (from 1141 ±143 pg/ml to 628 ±105, 593 ±69 pg/ml respectively). Chemical insult-increased IL-8 in SAC cultures provides an assay for screening countermeasures against the inhalation toxicity of chemical threat agents. The increase in the inflammatory cytokine IL-8 by both SM and phosgene may further provide common pharmacological targets for drugs with Multi-Threat Medical Countermeasure (MTMC) action against distinct chemical threats.

INTRODUCTION

Inflammation and associated inflammatory molecules such as IL-8 are implicated in the toxicity of the blister agent sulfur mustard (2,2'-dichlorodiethyl sulfide, SM) and edemagenic agent phosgene (carbonyl chloride) (1,2,3,4). Increased levels of the rodent equivalents to IL-8, NAP-1 in the skin of rabbits exposed to SM and MIP-2 in lungs of mice following inhalation exposure to phosgene, have been reported (5,6). The anti-inflammatory drug ibuprofen has shown efficacy for phosgene lung injury in rodents (reviewed, 5). Drugs with demonstrated efficacy against SM toxicity in the mouse ear vesicant model (MEVM) predominantly have anti-inflammatory action. Many of these drugs also inhibit SM-increased IL-8 in human epidermal keratinocyte (HEK) cultures (reviewed 3,7,8).

The Multi-Threat Medical Countermeasure (MTMC) hypothesis has been proposed with the aim of developing a countermeasure drug with efficacy against the inflammatory pathology caused by different classes of chemical threats (3). Experimental evidence supports the MTMC concept: [a] serine protease inhibitors can prolong the survival of animals intoxicated with the

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nerve agent soman and also protect against vesication caused by the blister agent SM (7,9); [b] poly(ADP-ribose) polymerase (PARP) inhibitors can reduce both soman-induced neuronal degeneration and SM-induced epidermal necrosis (7,10); and [c] the surfactant Curosurf® improves survival and decreases pulmonary edema for early-stage acute respiratory distress syndrome (ARDS) caused by perfluoroisobutene, lipopolysaccharide, and SM (11). The sites and mechanisms of action and the pathologies caused by different chemical insults vary; however, common biochemical signaling pathways or molecular mediators such as IL-8 provide targets for MTMC drugs (3). Even when threats extend to mixtures of agents and toxic industrial chemicals (TICs), or when nontraditional threat agents (NTA) evolve, such biochemical signaling pathways may remain constant and amenable to medical intervention via the MTMC concept.

SM–increased IL-8 in HEK cultures is a biomarker for cutaneous inflammation that has been used as an *in vitro* assay for screening anti-vesicant drugs (2,3,8). The mechanistic and drug screening precepts for cutaneous SM injury ascribed to SM-increased IL-8 in HEK cultures might be extended to SM and phosgene exposed SAC cultures. SM- and phosgene- increased IL-8 in SAC cultures could provide a biomarker for pulmonary inflammation associated with respiratory injury. SM- and phosgene- increased IL-8 in SAC cultures could further provide an *in vitro* drug-screening assay for countermeasures against inhalation of toxic chemicals.

METHODS

Reagents: Sulfur mustard (SM, 2,2'-dichlorodiethyl sulfide) with a purity of >98% was obtained from the U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD. Human lung epithelial small airway cells (SAC) and small airway growth media (SAGM™) and (growth factors) SAGM BulletKit® were purchased from CAMBREX, Walkersville, MD. Quantikine® Human IL-8 Immunoassay Kit was purchased from R&D Systems Inc., Minneapolis, MN.

Ibuprofen Preparation: Ibuprofen sodium salt, FW 228.3 (Sigma-Aldrich Co. St.Louis MO), was diluted in 0.9%, sterile preservative free saline solution (AmTech Group, Inc., Phoenix Scientific, Inc. St.Joseph, MO). The pH of the drug was adjusted with 0.25M HCl and 1M NaOH to pH 7.2 and filter sterilized with a StarLB 0.45 micron filter (Costar Corporation Cambridge MA). Ibuprofen was added to SAC cultures exposed 30 min previously to 2 ppm • min phosgene. Final drug concentrations of 1000, 500, 250, 125 and 62 μM were selected based on ibuprofen efficacy against phosgene toxicity in rodent *in vivo* studies (reviewed, 5).

Cell Culture: SAC were incubated at 37^o C in a 5% CO_2 incubator using standard procedures provided by the supplier. Subculturing to 24-well tissue culture plates was done using CAMBREX trypsin-EDTA.

Sulfur Mustard Exposure: SAC at 100% confluence were exposed to 25 to 400 μM SM in 1 ml KGM per well. The plates were maintained at room temperature in a fume hood for 1 hr to allow venting of volatile agent and then transferred to a 5% CO_2 incubator at 37^o C for a total incubation time of 24 hr. The same KGM medium was used for the entire 4 days of growth phase and confluent SAC culture. This resulted in accumulation of background levels of IL-8 in control cultures (1509 $\pm$ 128 pg/ml).

Phosgene Exposure: SAC at 100% confluence were exposed to 0.1 to 6.4 [ppm • min] of phosgene. The culture medium was removed from the well and tape (TimeMed Labeling Systems, Inc., Burr Ridge, IL) placed over the well. Phosgene (Matheson Tri-Gas Inc., Joliet, IL) was injected by syringe (Hamilton Company, Reno Nevada) punctured through tape, the tape was

removed after one minute and 1 ml of media restored. Phosgene was 10% phosgene, 90% nitrogen, certified mixture grade. Syringe was a Hamilton Gas Tight Syringe #1705 for 50 µl size with removable side port needle 22 gauge for 50 ul size. Exposures were done at room temperature in a fume hood (Kewaunee Scientific Corporation, Statesville, NC). Following exposure, cells were transferred to a 5% CO_2 incubator at 37^o C for a total incubation time of 24 hr. The SAGM medium was replaced with fresh media at the time of phosgene exposure on day 3 when SAC cultures were 100% confluent. SAC culture conditions of 1 day at 100% confluence resulted in insignificant background levels of IL-8 in controls (31 $\pm$ 33 pg/ml).

IL-8: IL-8 was assayed by the quantitative sandwich enzyme technique described in R&D Systems Inc. (Minneapolis, MN) pamphlet (Quantikine® Human IL-8 Immunoassay).

Data Analysis: A one-way analysis of variance (ANOVA) was used for each group, SM, phosgene, and ibuprofen, to compare the doses. If there was a significant dose effect, then a Dunnett's test was used to compare doses with the control, 0 dose. Statistical significance was defined as $p<0.05$. Data are presented as means and standard error of mean (SEM), N = 4.

RESULTS

The SAC negative control background IL-8 levels were significantly higher for SM exposures (1509 $\pm$ 128 pg/ml) compared with the phosgene exposures (31 $\pm$ 33 pg/ml) because of the different cell culture conditions described in Methods. Therefore, SM-increased IL-8 is expressed as total IL-8 minus the accumulated background and phosgene-increased IL-8 as total IL-8. SAC cultures exposed 24 hr previously to 25, 50, 100, 200 or 400 µM SM demonstrated significantly increased IL-8 to all exposures except the 400 µM high dose (Figure 1). The intermediate exposures of 100 µM SM gave maximum SM increased-IL-8 (1013 $\pm$123 pg/ml) above background levels. High doses of 200 and 400 µM SM progressively inhibited IL-8.

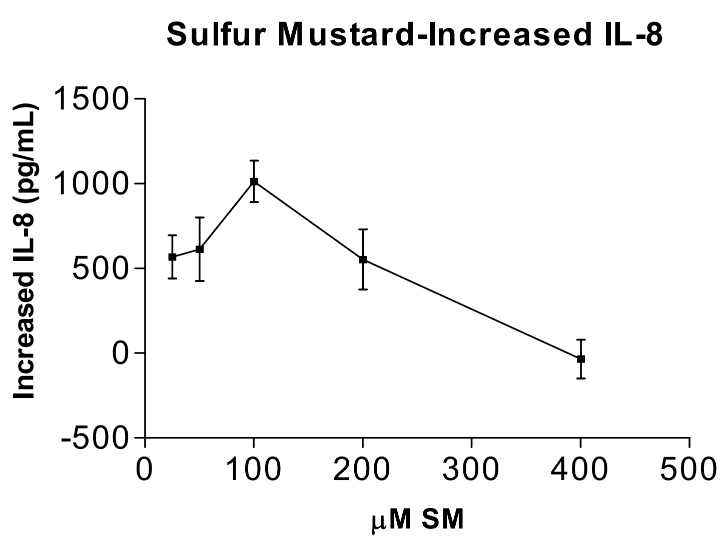

Figure 1. SM-increased IL-8 in SAC cultures. SAC cultures exposed 24 hr previously to 25, 50, 100, 200 or 400 µM SM demonstrated significant SM-increased IL-8 to all exposures except the 400 µM high dose. SM-increased IL-8 was calculated as total IL-8 pg/ml minus background control IL-8 (1509 pg/ml). Results are presented as mean $\pm$ SEM, N= 4. Statistical significance is defined as $p<0.05$.

SAC cultures exposed 24 hr previously to 0.1 0.2. 0.4, 0.8, 1.6, 3.2 or 6.4 ppm • min phosgene for 1 min. demonstrated significantly increased IL-8 at all exposures except 0.1 ppm • min. low dose. The intermediate exposures of 1.6 ppm • min phosgene gave optimal increases in IL-8 (965 $\pm$181 pg/ml). Higher exposures at 3.2 and 6.4 ppm • min phosgene progressively inhibited IL-8 from maximal levels (Figure 2).

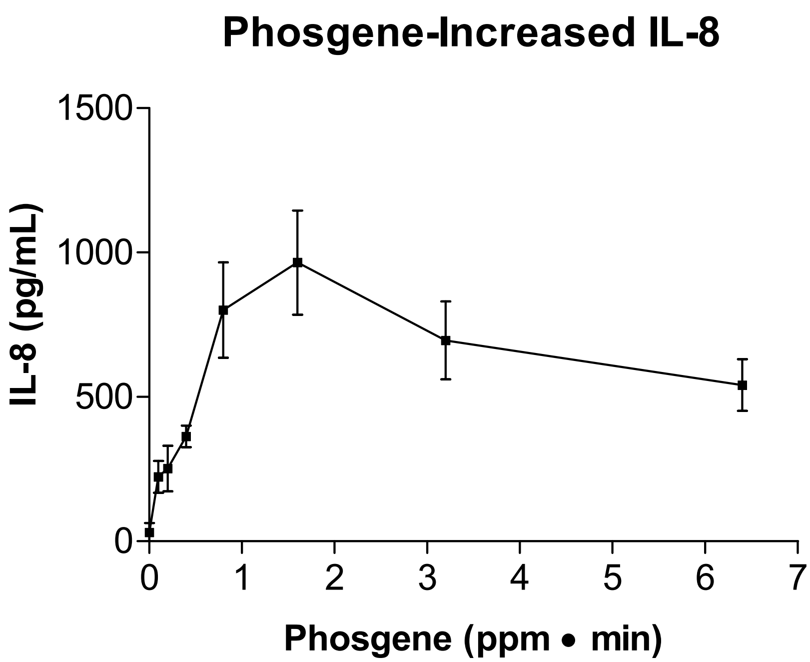

Figure. 2. Phosgene-increased IL-8 in SAC cultures. SAC cultures exposed 24 hr previously to 0.1 0.2. 0.4, 0.8, 1.6, 3.2 or 6.4 ppm • min phosgene demonstrated significantly increased IL-8 at all exposures except 0.1 ppm • min. Phosgene increased IL-8 was calculated as total IL-8 pg/ml. Results are presented as mean $\pm$ SEM, N= 4. Statistical significance is defined as $p<0.05$.

Ibuprofen significantly inhibited 2 ppm • min phosgene–increased IL-8 at all the tested doses. Maximum efficacy of nearly 50% inhibition was seen at the 125 and 250 μM doses (from 1141 $\pm$143 pg/ml to 628 $\pm$105, 593 $\pm$69 pg/ml respectively, Figure 3). Higher than optimal exposure to either SM or phosgene increased cytotoxicity as measured by trypan blue exclusion and decreased IL-8 levels (data not shown).

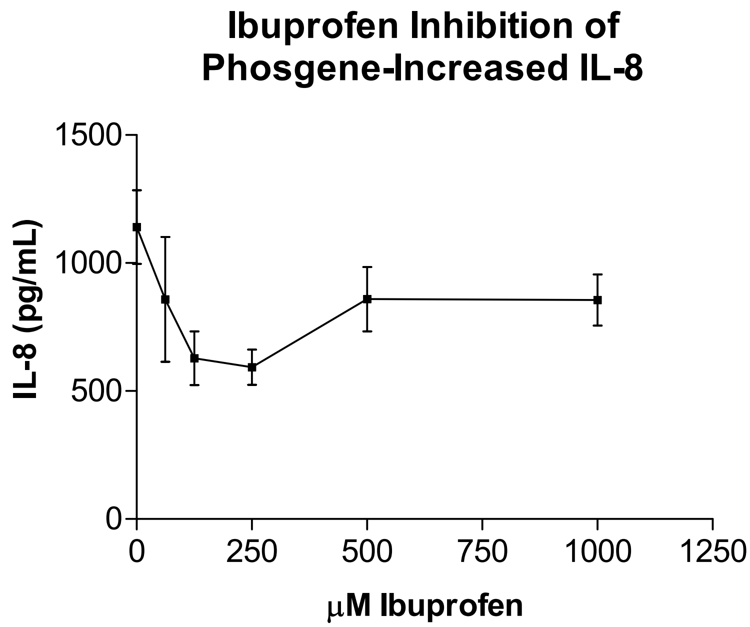

Figure 3. Ibuprofen inhibited phosgene-increased IL-8 in SAC cultures. Ibuprofen was added to SAC cultures exposed 30 min. previously to 2 ppm • min phosgene. Final drug concentrations were 1000, 500, 250, 125 and 62 μM. Ibuprofen at all doses tested significantly inhibited phosgene–increased IL-8 at 24 hr. after phosgene exposure. Phosgene increased IL-8 was calculated as total IL-8 pg/ml. Results are presented as mean $\pm$ SEM, N= 4. Statistical significance is defined as $p<0.05$.

DISCUSSION

Human SAC cultures exposed to either SM [25 to 400 μM] or phosgene [0.1 to 6.4 ppm • min] demonstrated significantly increased IL-8. A maximum increase of about 1000 pg/ml IL-8 in SAC cultures exposed to either phosgene or SM was observed (Figures 1 and 2). The pattern of IL-8 response, increase to maximum levels followed by inhibition at higher doses, was similar for both agents. Ibuprofen has shown to be efficacious against phosgene pulmonary toxicity in rodents (reviewed, 5) and some efficacy in the MEVM (personal communication, Dr. William J. Smith, USAMRICD, APG Md). Ibuprofen (62, 125, 250, 500, 1000 μM) significantly diminished phosgene-increased IL-8 in SAC cultures exposed to 2 ppm • min phosgene (Figure 3). Furthermore, the doses of ibuprofen that decreased phosgene-increased IL-8 about 50% in SAC also inhibited SM-increased IL-8 in HEK cultures to about the same extent (unpublished observation).

The potential relationship of the present studies on SM- and phosgene- increased IL-8 in SAC culture to previous work on SM cutaneous injury should be considered. The anti-inflammatory actions of anti-vesicant compounds result in inhibition of SM-increased IL-8 in HEK and efficacy in the MEVM. Screening by our Institute of more than 400 compounds in the MEVM for cutaneous SM injury has yielded 19 compounds that reduced SM histopathology greater than 50% (7,12,13). These 19 compounds include 7 listed as anti-inflammatory drugs, consisting of 5 capsaicin analogues, a single cyclooxygenase (COX) inhibitor, indomethacin and a calmodulin antagonist, fluphenazine (7,13). The 3 protease inhibitors and 3 PARP inhibitors

included in the list of anti-vesicant drugs also have the potential to inhibit inflammatory responses (4,8,14). The remaining 6 anti-vesicant drugs, Na 3-sulfonatopropyl glutathionyl disulfide, 3% hydrogen peroxide gel, dimercaprol, and 3 other mercaptopyridines anaologues, are listed as SM scavengers (7). However, mercaptopyridine compounds have demonstrated anti-inflammatory pharmacology to include inhibition of the cellular release of the inflammatory cytokine IL-1 (15). Therefore, with the exceptions of two of the listed SM scavengers, Na 3-sulfonatopropyl glutathionyl disulfide and 3% hydrogen peroxide gel, the remaining 17 compounds with efficacy against SM toxicity can have anti-inflammatory pharmacology. In previous studies and despite diverse pharmacology, the antivesicant drugs, protease inhibitors, the COX inhibitor indomethacin, the PARP inhibitor 3-(4'-Bromophenyl)ureidobenzamide and the calmodulin antagonist fluphenazine, each significantly suppressed the inflammatory cytokine IL-8 in SM-exposed HEK cultures (3,8). Furthermore the two selected lead compounds in the MEVM that are anologues of mercaptopyridine and capsaicin completely suppressed SM-increased IL-8 in HEK cultures (unpublished observation).

The *in vitro* and *in vivo* inflammagenic actions of SM and efficacy of a preponderance of drugs with anti-inflammatory actions in the MEVM and HEK cultures strongly implicates inflammation as a major component of SM cutaneous toxicity. These precepts may also extend to predicting mechanisms of and drug efficacy against inhalation toxicity, as exemplified by SM- and phosgene- increased IL-8 in SAC culture.

CONCLUSIONS

The blister agent SM and the edemagenic agent phosgene increased IL-8 in SAC cultures, similar to increases previously reported in SM-exposed HEK. Increased IL-8 in SAC cultures as a biomarker for inflammation may reflect mechanisms of toxicity and further provide an *in vitro* screening assay for medical countermeasures against inhalation toxicity of chemical threat agents. Inflammation may further provide a common target for Multi-Threat Medical Countermeasure against distinct chemical threats such as phosgene and SM.

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